



Monitoring antibody–antigen reactions at conducting polymer-based immunosensors using impedance spectroscopy

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Received 25 November 1998; received in revised form 4 June 1999

Abstract

The mechanisms of antibody–antigen (Ab–Ag) interactions at conducting polypyrrole electrodes have been investigated using impedance spectroscopy techniques. The effects of the variation in ion exchange, solution composition, and the condition of the synthesis have been used to study the capacitive behavior of antibody-containing polypyrrole electrodes in the presence of the antigen. The theory of charge generation and transportation in the heterogeneous polymeric domains is proposed as the predominant basis for the analytical signals observed at these electrodes. The significant difference observed in the impedance response at different potentials confirmed that the Ab–Ag interaction was largely influenced by the applied potential. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Mechanism; Charge generation; Charge propagation; Antibody–antigen reactions; Conducting polymers; Immunochemical sensor

1. Introduction

The electrochemical modification of conducting polymer electrodes using biochemical reagents is a simple step that can be used to generate useful analytical signals [1,2]. The basis for using conducting polymers for sensing purposes lies in their ability to be reversibly oxidized and/or reduced through the application of electrical potential [3]. Conducting electroactive polymers (CEPs) that contain bioactive molecules, e.g. enzymes, antibodies, cells, and DNA have been used extensively in biosensor applications [2–4]. In addition, the mechanism of signal generation at conducting polymers using enzymes and low molecular weight molecules has previously been examined [5–8]. How-

ever, to our knowledge, no similar study has been carried out that involves the incorporation of antibodies. Essentially, previously reported studies have focused on the electrochemistry of antibody-containing conducting polymers in which the use of pulsed amperometric detection was found to be suitable for generating antibody–antigen (Ab–Ag) signals [9,10].

In this work, we report how we have attempted to improve our understanding of the kinetics and mechanism of signal generation using anti-human serum albumin (AHSA) antibody incorporated into a polypyrrole film and its corresponding antigen (HSA). The binding event between a CEP-immobilized antibody (Ab) and its antigen (Ag) are each characterized by a change in the double layer capacitance (C_{dl}) and the charge transfer resistance (among other obtainable variables) as a function of frequency. This paper shows the use of the ac impedance technique for the study of the resistivity of AHSA-containing polypyrrole electrodes. In addi-

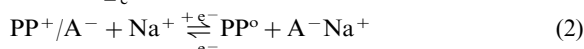
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tion, the paper proposes the theory of charge generation and transportation in the heterogeneous polymeric domains as the predominant basis for the analytical signals observed at these electrodes. The effects of the variation in ion exchange, solution composition, and the condition of the synthesis have been used to examine the capacitive behavior of AHSA-containing polypyrrole electrodes in the presence of the antigen.

2. Mechanism of charge generation and propagation during Ab–Ag binding at PP/AHSA electrodes

Previous workers have shown that doping polypyrrole with low-molecular-weight anions makes it act as an insulator at cathodic potential where a kinetic-controlled electrode process is predominant [5,7]. However, at anodic potential, the electrochemistry of the polymer is diffusion-controlled, where ions diffuse into the films. In addition, anion movement is rarely the sole source of charge balance. In some cases, cation movement into the polymer can also occur according to:



where PP^+ and PP^0 represent the oxidized and reduced forms of the polymer and A^- is the counterion dopant.

Eq. (2) can be predominant if the counterion is the large, highly resistive biochemical species such as an antibody. Under these circumstances, the mechanism of charge transport appears to be more complicated than mere simple ion-exchange process. Previously, we have studied the electrochemistry of antibody-immobilized polypyrrole electrodes and found that the incorporated antibody was not released during electrochemical switching even at cathodic potential, e.g. at -1.00 V versus Ag/AgCl [9,10]. Rather, we found that charge balance was kept through the incorporation of cations of the supporting electrolyte into the reduced electrode where it interacted with the negatively charged antibody dopant. Consequently, several factors contribute to the current observed at the PP/AHSA electrode in the presence of the HSA antigen. These include the IR drop at the PP/solution interface, the migration current from the bulk of the polymer membrane (since the polymer is a binary electrolyte), and the adsorption of antigen onto the antibody, which depends on the concentration of the HSA. Due to the foregoing reasons, we propose a mechanism of charge generation and propagation in the heterogeneous polymer interface to explain the current flow at the PP/AHSA surface during Ab–Ag binding. According to this mechanism, the current obtained at the antibody-immobilized conducting polymer electrodes occurs via the following steps:

1. Diffusion of ions to the electrode
2. Charge transfer at the porous PP/membrane interface
3. Migration through the polymer membrane, and
4. Adsorption/desorption of the Ag at the PP/solution interface

The slow rate of adsorption/desorption process in step 4 is considered to be the rate-determining step. This step can be controlled through the appropriate choice of electrical potential. Results obtained using impedance spectroscopy indicated that the binding event was characterized by a change in the C_{dl} , while the charge transfer resistance, among other variables, was a function of frequency.

3. Experimental

3.1. Reagents

All solutions were made up in Nanopure, deionized distilled water ($17.5 \text{ M}\Omega/\text{cm}$). Anti-human serum albumin (AHSA), at 59 mg/ml total protein and 4 mg/ml specific antibody, was obtained from Sigma (A-7544). This was dialyzed against pH 7.2 phosphate buffer (PBS) before being frozen and stored in working aliquots. NaCl (99%) was purchased from Aldrich Chemicals and stored in a dessicator. In addition, HSA was purchased from Sigma (A-1887) and stored at 4°C . Pyrrole was obtained from Aldrich and was distilled before use. All references to PBS are at pH 7.2.

3.2. Instrumentation

The electrochemical instrumentation used for these experiments consists of an EG&G potentiostat/galvanostat (model 263A) and EG&G lock-in-amplifier (model 5210). Data collection and analysis were conducted using electrochemistry software model 270 and electrochemical impedance spectroscopy (EIS) software model 398. The lock-in-amplifier was used to generate frequencies above the 10 Hz level and the potentiostat was used to control the potential. Electrochemical experiments were conducted using a three-electrode cell with a BAS platinum disc as the working electrode (0.0201 cm^2). Potentials were measured relative to an aqueous, saturated Ag/AgCl double junction (reference electrode) and a platinum wire (auxiliary electrode).

3.3. Preparation of antibody-containing polypyrrole films

Polymers were prepared by galvanostatic polymerization as previously reported [9,10]. PP/AHSA films were grown in a solution containing 0.5 M pyrrole with varying concentrations of anti-HSA (i.e. 25, 50, 100 and

200 μ l) made up to a 10 ml volume of PBS. A current density of 0.3 mA was applied to the working electrode, which was immersed in the growing solution for 30 s. PP/PBS was grown using 0.5 M pyrrole with the PBS as the supporting electrolyte. All solutions were N_2 -purged for 10 min, and then blanketed for the remainder of the experiment. All freshly grown polymer films were characterized using cyclic voltammetry at a scan rate of 50 mV/s in 0.1 M NaCl. PP/AHSA films were characterized first in 0.1 M NaCl and then incubated in 0.1% BSA in the presence of the PBS solution at room temperature for about 30 min. This helps to eliminate non-specific adsorption. The blocked PP/AHSA electrodes were then immersed in 10 ml of 100 μ g/ml HSA in PBS solution during impedance analysis. The overoxidized PP/AHSA film was incubated in the same HSA solution overnight at 4 $^{\circ}$ C, rinsed well and the impedance spectra were recorded in PBS electrolyte. PP/PBS films were used as control and subjected to the same conditions of analysis as specified for the immunosensing electrodes.

3.4. Impedance analysis

Impedance scans were conducted within the frequency range 100 kHz to 100 mHz at a voltage amplitude of 15 mV (rms). Impedance spectra were recorded at bias voltages of -0.6 , -0.4 , -0.2 , 0 , 0.2 and 0.4 V with a pre-equilibration time of 30 s. From the Bode and Nyquist plots generated, the impedance parameters obtained include the C_{dl} , the uncompensated ohmic resistance (R_s), the charge transfer resistance (R_{ct}), the depression angle (θ), and the switching frequencies. The switching frequency is defined as the frequency where the complex-plane impedance spectrum changes from kinetic control to diffusion control.

4. Results and discussions

4.1. Polymerization and incorporation of AHSA

Polymers were synthesized as described in Section 3. The supporting electrolyte used in all of the experiments was phosphate buffer at pH 7.2. A cyclic voltammetry experiment was initially performed on each freshly-made film to determine the effect of antibody concentration on the electroactivity of the polypyrrole. This involved growing the polypyrrole electrodes in different concentrations of AHSA and measuring the electroactivity of the resulting films in 0.1 M NaCl. The potential was scanned between -0.8 and $+0.6$ V. Typical behavior of AHSA-containing polypyrrole electrodes (PP/AHSA) in PBS buffer is shown in Fig. 1. The CV data indicates that the polymer film is electroactive having a prominent anodic peak at 0 V as well as the characteristic broad reduction peak. A change in the antibody concentration did not produce a significant change in the electroactivity of the PP/AHSA. This type of behavior is similar to those observed during previously reported polypyrrole films containing low-molecular-weight counterions [12–15].

4.2. Impedance spectroscopy of AHSA-containing polymers

Classical thermodynamic concepts have been applied extensively to the characterization of Ab–Ag interactions under equilibrium conditions [16,17]. These have not adequately explained the non-equilibrium kinetics of the Ab–Ag interaction at organic conducting polymer interfaces. To obtain the kinetic and mechanistic contributions to the analytical signal at CEP surfaces, an in-situ, non-destructive, electrochemical technique is

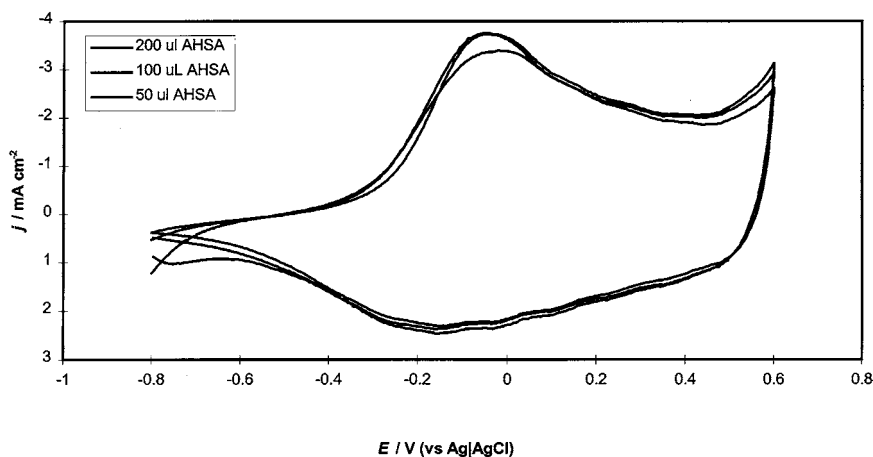


Fig. 1. Cyclic voltammograms obtained for antibody-immobilized polypyrrole electrodes grown with varying solution antibody concentrations. Scan rate = 50 mV/s, supporting electrolyte = PBS buffer, pH 7.2.

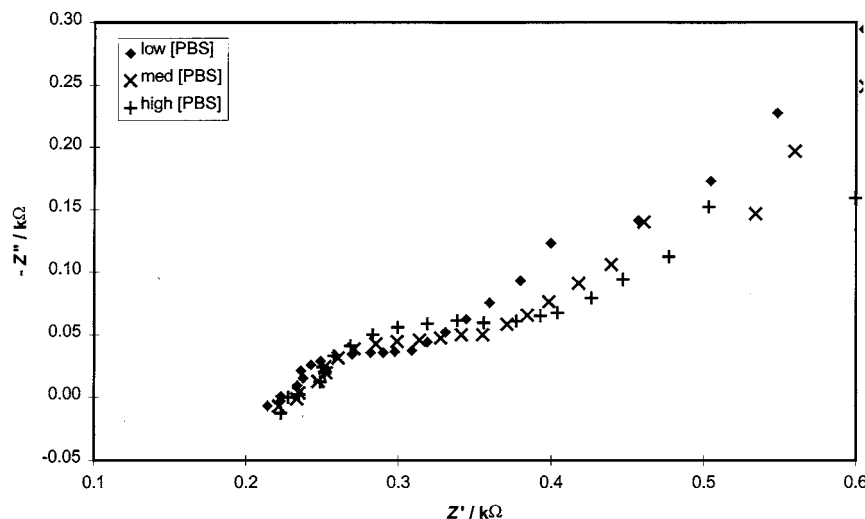


Fig. 2. Effect of low, medium and high phosphate concentration on impedance behavior of a PP/AHSA film at 0 V vs. Ag | AgCl.

desirable. We have chosen the use of EIS because it allows the resolution of both the kinetic and/or the transport processes occurring within the system at various applied potentials. This involves the use of minimal ac perturbation to determine the ionic and/or the electronic transport phenomena. Since EIS can be used in the case of highly resistive solutions, the determination of the transport process will not be limited to surface reaction. Sensor characterization using EIS over a wide range of frequencies and applied potentials can provide a variety of information on the non-faradaic processes. These include the double-layer capacitance C_{dl} , solution resistance, and diffusion behavior in the low-limit frequency region. Faradaic processes can be probed by extracting the charge-transfer resistance R_{ct} , under various applied potentials and film conditions.

4.2.1. Effect of phosphate ion concentration

First, impedance spectroscopy of a PP/AHSA electrode was carried out in a buffer solution to determine if any change in the phosphate concentration influenced the charge transfer within the film. Impedance analysis was performed at a bias potential of 0 V at a frequency range of 100 kHz to 100 mHz at 5 mV amplitude. The bias voltage was set at 0 V because at this voltage, the voltammetric data exhibits an anodic peak. A pre-equilibration time of 2 min was set to allow the electrode to equilibrate prior to recording spectra in low, medium and high PBS concentrations. Concentration of phosphate ion was varied by maintaining the ratio between the acid–base pair constant at approximately 6.05 and changing the total amount of phosphates to half (for low PBS), or by doubling the original PBS medium (for high PBS). NaCl and KCl in the PBS solutions were kept constant at their original concentrations. Hence,

any change observed in the impedance spectra from low to high phosphate concentration is attributed to only the phosphate species present in the electrolyte.

Fig. 2 shows the overlaid impedance spectra for the varying PBS concentrations. From these data, it is clear that an increase in phosphate concentration results in a corresponding increase in R_{ct} , which can be extrapolated from the second x-intercept of the fitted semicircle. R_{ct} values recorded for the low, medium and high PBS concentrations were 83.7, 101.3 and 195 Ω , respectively. The observed behavior might be attributed to the possibility of the phosphates undergoing adsorption or diffusion into the film at 0 V. In addition, a general trend was observed in the slope of the linear region, which represents the diffusion-controlled portion of the spectra. As the phosphate concentration decreases, the slope values increased to 0.529, 0.822 and 0.882 Ω/Ω for high, medium and low phosphate concentration, respectively. This observation is consistent with heterogeneities in the electrode material or non-uniform diffusion observed in the case of inhomogeneous materials [18]. Therefore, it is possible that the adsorption of phosphates species to the PP/AHSA film hinders, in some way, the normal diffusion of other ionic electrolyte species (e.g. Na^+ , Cl^-) within the film. At 0 V, phosphate diffusion and/or adsorption to the PP film provides resistance to charge transfer at this active potential as outlined in the mechanistic steps above. After the impedance analysis, the PP/AHSA films were recharacterized in 0.1 M NaCl to determine if electroactivity was maintained throughout the analysis. In Fig. 3, the PP/AHSA film shows little reduction in electroactivity, even after exposure to varying levels of phosphate in solution. Consequently, the electrochemistry of the film is influenced by adsorption to the film surface, which hinders electron transfer.

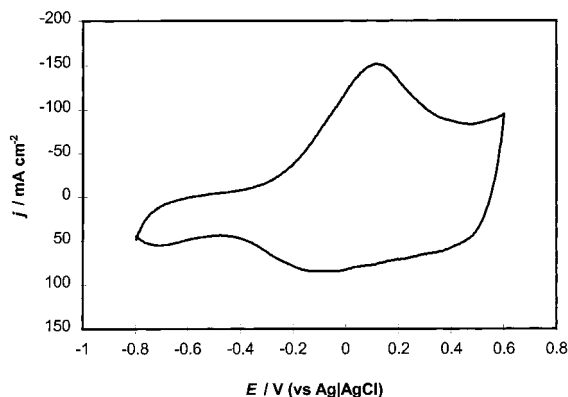


Fig. 3. Cyclic voltammogram obtained for the PP/AHSA in 0.1 M NaCl after impedance analyses in solutions of varying phosphate concentration.

4.2.2. Impedance measurements at PP/AHSA electrodes during HSA binding

The impedance study of PP/AHSA was carried out to establish the influence of applied potential on C_{dl} , R_{ct} , and exchange current densities during HSA binding. A polypyrrole film grown in PBS buffer was used as the blank (PP/PBS). Parallel studies using a PP/AHSA electrode were conducted to establish the extent of charge transfer resistance in the Ab-immobilized

films. Fig. 5(a) is a Bode plot generated at a PP/PBS electrode when scanned in PBS. At anodic potentials, the polymer conformed to the expected electrochemical switching through the diffusion of anions into the film, thus resulting in desirable conducting properties and low R_{ct} . On the contrary, at sufficiently negative potentials (e.g. -0.6 V), PP/PBS film exists in its reduced state; therefore charge transfer is largely limited by electrode kinetics. Consequently, the Bode plot at -0.6 V exhibits a much higher charge transfer resistance than at the anodic potential. At anodic potentials, the PP/PBS acts as an infinite capacitor and charge transfer resistance is not observed. This is synonymous with the expected polypyrrole behavior when doped with low-molecular-weight anions [6,7,19].

The impedance spectra of a typical PP/AHSA electrode indicates a switch from a kinetically-controlled process at high frequencies to diffusion-controlled behavior at low frequencies, as shown in Fig. 4. The switching frequency can be used as a comparative measure to gauge the relative electroactivities of different polypyrrole films, since the second semicircle intercept will fall at different ohmic values ($R_s + R_{ct}$) with a change in film conductivity. Therefore, we would expect that the switching frequency of a PP/AHSA electrode will be higher at more anodic potentials (due to lower R_{ct}) and lower at reducing potentials. This behavior is expected because the electrode becomes increasingly

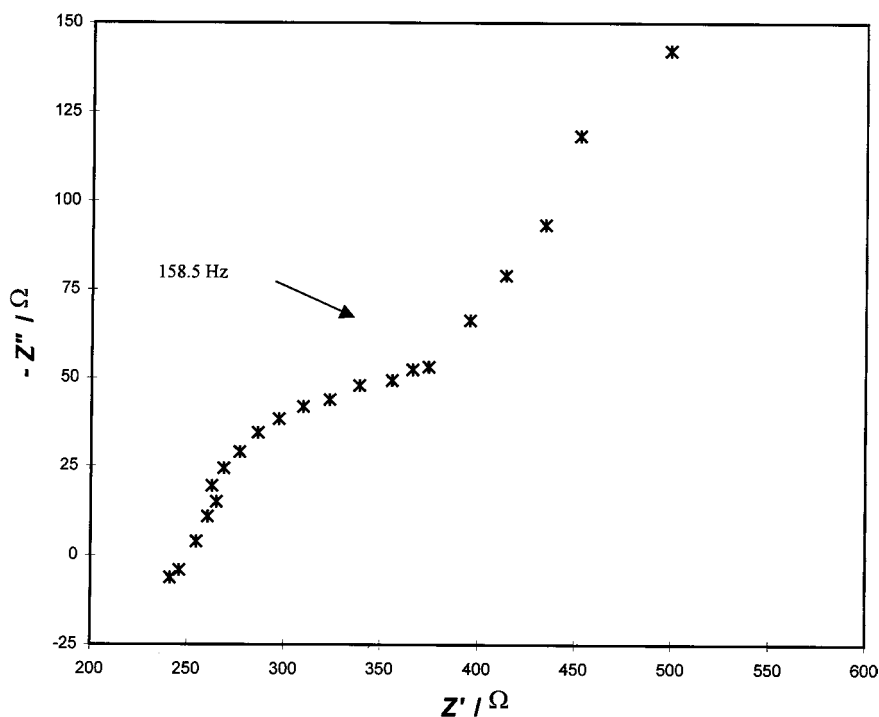


Fig. 4. A PP/AHSA film undergoing a switch from kinetic to diffusion control in PBS at a switching frequency of 158.5 Hz. Bias potential = 0 V, amplitude = 5 mV rms.

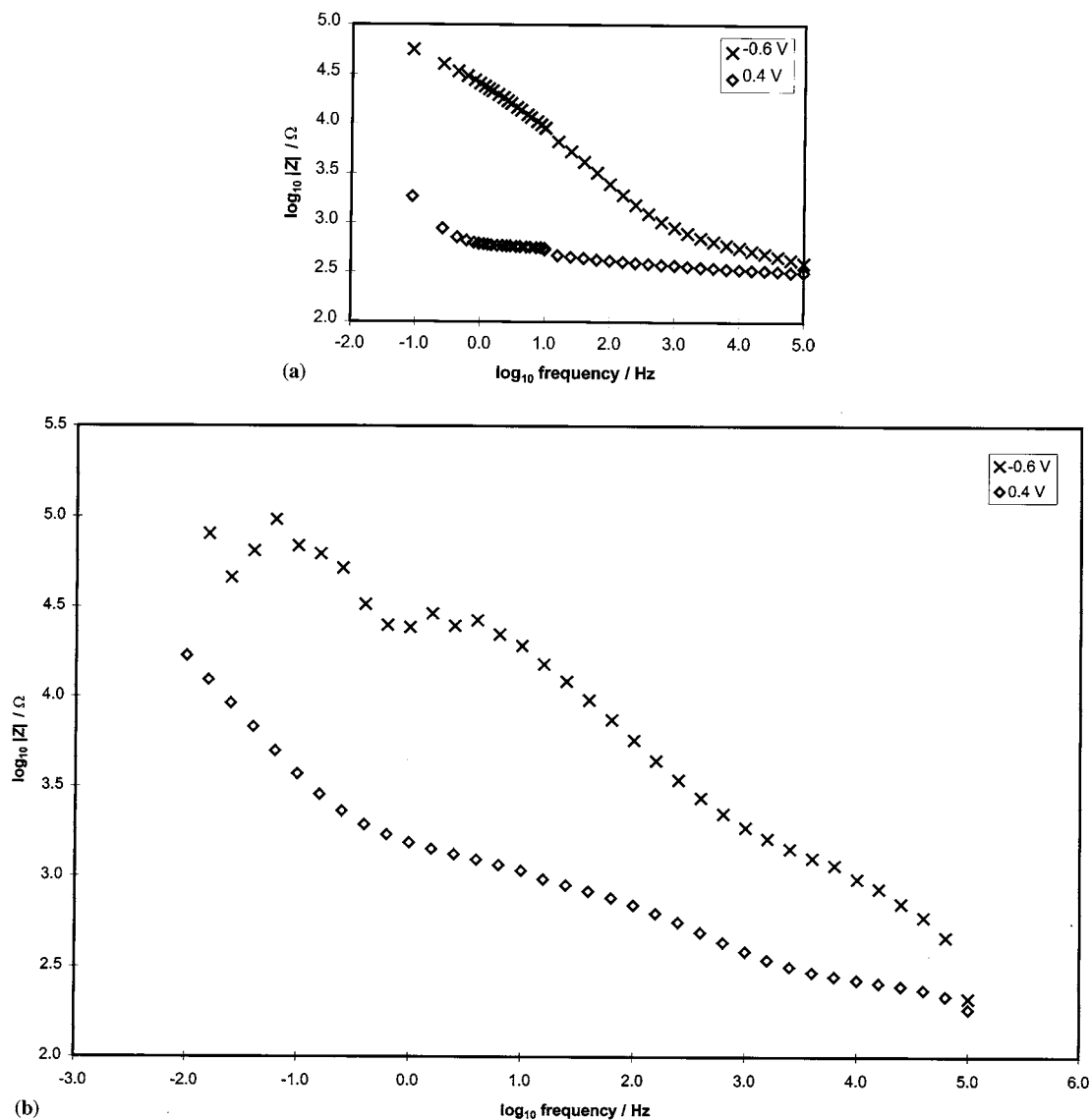


Fig. 5. (a) Bode plot impedance of PP/PBS in PBS at -0.6 and 0.4 V vs. Ag|AgCl. (b) Bode plot impedance of PP/AHSA in PBS at -0.6 and 0.4 V vs. Ag|AgCl. (c) Bode plot impedance of PP/AHSA in HSA solution at -0.6 and 0.4 V vs. Ag|AgCl.

electroactive as the potential changes toward positive values. Therefore, the reactivity of the electrode depends on the diffusion of charge within the polymer. The high R_{ct} observed is probably due to the inability of the entrapped biomolecules to diffuse within the film or to allow cation/anion exchange with the solution to balance film charge. This is contrary to PP/PBS electrodes, which exhibit infinitely high capacitance at anodic potentials in the low frequency limit.

PP/AHSA exhibits two time constants as shown in Fig. 5(b). Upon exposure to PBS electrolyte containing HSA, the two time constants are not preserved at either -0.6 or 0.4 V as shown in Fig. 5(c). The time constants presumably

represent the diffusion of ions to the film and adsorption of HSA at the interface. Moreover, extraction of C_{dl} from the Bode plots show that C_{dl} decreases beyond 0 V only in the presence of HSA. This potential region signifies the onset of Ab–Ag binding at the PP/AHSA surface [3,11]. This observation is consistent with the proposed mechanism of charge transfer and migration at the porous polypyrrole membrane.

Fig. 6 shows the results obtained for charge transfer dynamics at an overoxidized PPy/AHSA film. Results of the CV experiments confirmed the overoxidation of the film as a marked loss in redox peaks. It is clear that there is more similarity in the impedance spectra at negative and positive potentials in the overoxidized film

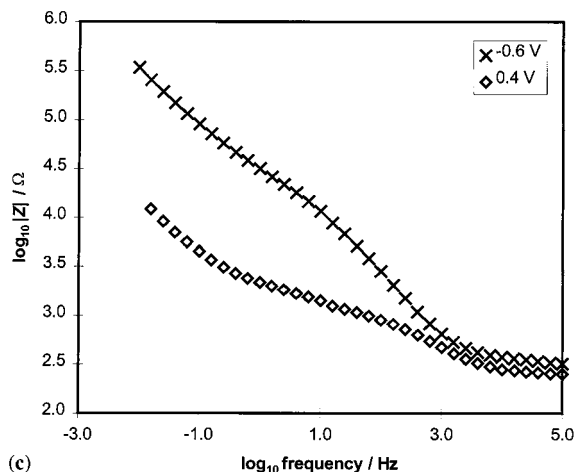


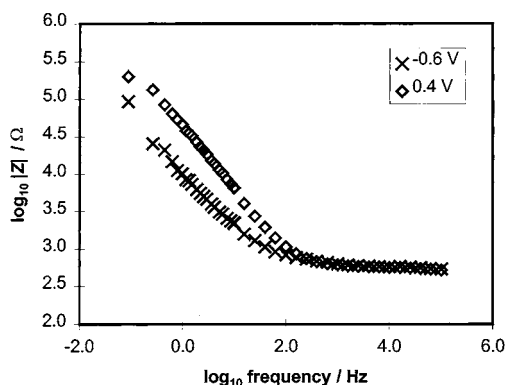
Fig. 5. (Continued)

than in the electroactive films. As expected the presence of AHSA in the overoxidized polymer film introduces a considerable charge transfer resistance because overoxidation does not allow adequate charge-balancing by either cation/anion uptake from solution or film counterion expelling into solution. Whether the overoxidized film is held at negative or positive potential has less effect on electroactivity than for a conducting film because the limiting factor would be the presence of adsorbed biomolecules, yielding the high R_{ct} values observed. Fig. 7 is a bar chart showing relative R_{ct} values extracted from Nyquist plots at three significant applied potentials. Very high R_{ct} values for the overoxidized film is expected because the film becomes less conductive due to destruction of the 2D and 3D electron conducting pathways by excess influx of positive charge.

4.2.3. Influence of applied potential on θ

It is possible to use the variation in θ with potential to study the surface inhomogeneity of the sensing electrodes. Here, the angle of depression is denoted as negative, since the angle is measured beneath the x -axis to the center of the circle. Therefore, a more negative angle signifies greater semicircle depression. Table 1 shows how θ varies at the sensing electrodes compared to the blank polymer. Generally, for all films with the exception of PP/AHSA in HSA solution, θ at -0.6 V is the greatest in the series where the most depression occurs. Evidently the polymer films behave quite differently at -0.6 V (negative potentials) than at positive potentials. At negative potentials, the polypyrrole undergoes a reduction during which neutral domains are first formed at the Pt/PPy interface. Afterwards, additional reduction of the polymer domain further out toward the solution is more difficult because the charge must be transported from the Pt/PP interface through

non-conductive, neutral domain to the outlying regions. This results in a very high R_{ct} value, hence the distribution of the relaxation times for this single process and the depressed semicircle in the PPy/AHSA can be attributed to an inhomogeneous electrode surface. This phenomenon has previously been observed in the other polypyrrole films [20,21]. An increase in applied potential, which causes greater depression, supports the theory of charge generation and propagation in the heterogeneous polymeric domains rather than through the bulk of the polymer. Compared to the PP/AHSA in PBS, all θ are smaller (semicircle is less depressed) when in the presence of HSA and θ is least when the potential is -0.6 V. This may correspond to a conformational change in the polymer matrix from the accommodation of HSA by the film and therefore a more restricted (less heterogeneous) matrix. The angle of depression decreases between -0.4 and -0.2 V for PP/AHSA in the presence of HSA; this

Fig. 6. Bode plot impedance of overoxidized PP/AHSA-HSA in PBS at -0.6 and 0.4 V vs. Ag|AgCl.

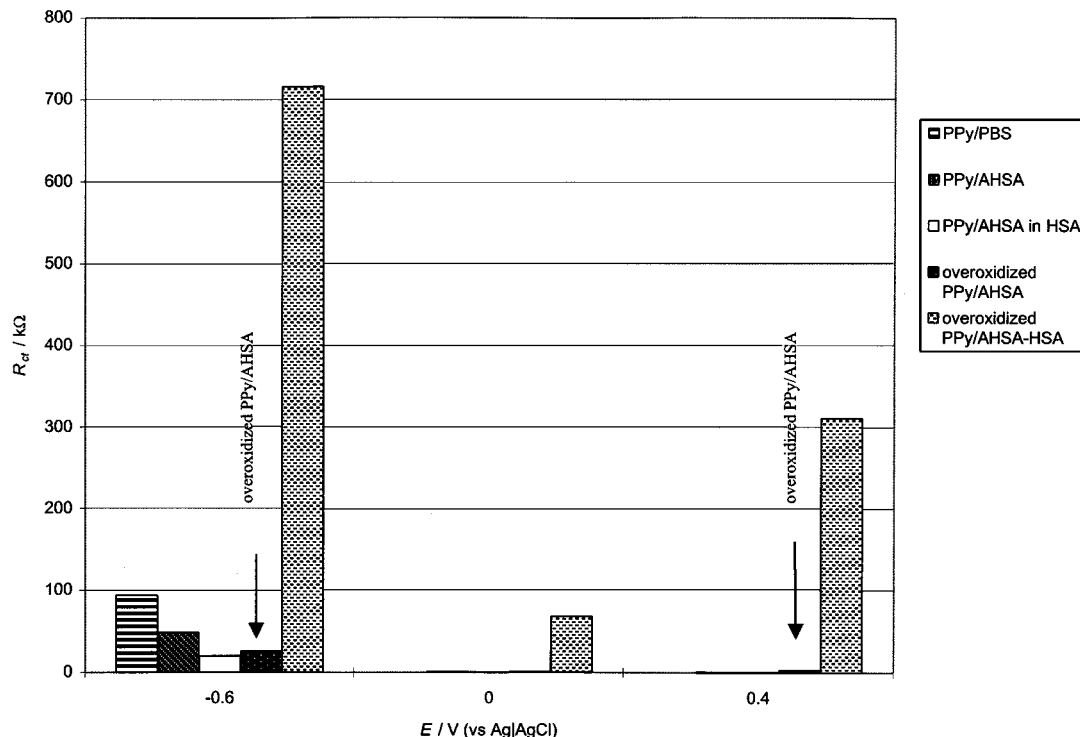


Fig. 7. Bar chart exhibiting change in R_{ct} with applied potential for all films studied.

potential range presumably precedes the potential region for the onset of AHSA–HSA binding. Furthermore, due to its large size and deeply folded nature, Ab cannot be expelled as the film is held at oxidizing potentials. Beyond -0.4 V, θ gradually increases, inherently increasing sensor response and leveling off due to saturation. The significant difference observed in the impedance response at different potentials confirms that the Ab–Ag interaction was largely influenced by the applied potential. This agreed with theoretical inferences drawn earlier.

5. Summary and conclusions

Impedance spectroscopy technique has been used to study the mechanism of antibody-immobilized CEP electrodes. It was found that the mechanism of interaction strongly depends on the applied potential. This was important for predicting the behavior of Ab–Ag binding at these surfaces. From the equivalent circuit models, it was also found that charge transport through the films occurs via different pathways depending on the potential applied. It was found that at

Table 1

Values of depression angle (θ) with applied potential for polypyrrole films

	E/V (vs. Ag AgCl)					
	–0.6	–0.4	–0.2	0	0.2	0.4
PPy/PBS	–43.3	–24.5	N/A	N/A	N/A	N/A
PPy/AHSA in PBS	–34.5	–28.5	–31.4	–32.1	–34.1	–36.2
PPy/AHSA in HSA	–21.1	–25.1	–23.6	–24.9	–28.6	–27.5
Overoxidized PPy/AHSA	–40.7	–10.4	–14.6	–22.7	–20.8	–22.6
Overoxidized PPy/AHSA–HSA	–28.8	–12.4	–13.7	–16.3	–16.7	–13.8

slightly negative potentials, Ab–Ag interaction occurred with a considerable decrease in the capacitance. The theory of the charge transfer in this region was further supported by a decrease in θ , thus reflecting a shift in the matrix toward a least restrictive domain that would carry the charge. Results obtained are consistent with the proposed theory of charge generation and propagation in the heterogeneous polymer domain.

Acknowledgements

This work was supported by the US-EPA's Office of Research and Development.

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